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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/20, 1/21 // C11D 3/386 — A1

(11) International Publication Number: WO 97/41212

(43) International Publication Date: 6 November 1997 (06.11.97)

(21) International Application Number: PCT/DK97/00179

(22) International Filing Date: 22 April 1997 (22.04.97)

(30) Priority Data:

501/96	25 April 1996 (25.04.96)	DK
500/96	25 April 1996 (25.04.96)	DK

(71) Applicant (*for all designated States except US*): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): HIRAYAMA, Satoshi [JP/JP]; Estate-pia U2-202, 732, Matsugaoka 5-chome, Nagareyama, Chiba 270-01 (JP). TAIRA, Rikako [JP/JP]; 16-3, Minami-nagareyama 3-chome, Nagareyama-shi, Chiba 270-01 (JP). BORCH, Kim [DK/DK]; Vandtaarnsvej 18, DK-3460 Birkerød (DK). SANDAL, Thomas [DK/DK]; Sortemosevej 31, DK-2730 Herlev (DK). HALKIER, Torben [DK/DK]; Hestkoebvej 11E, DK-3460 Birkerød (DK). OXENBØLL, Karen, M. [DK/DK]; Slotsvej 76, DK-2920 Charlottenlund (DK). NIELSEN, Bjarne, Rønfeldt [DK/DK]; Ligustervaenget 27, DK-2830 Virum (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: ALKALINE LIPOLYTIC ENZYME

(57) Abstract

Lipolytic enzymes with high activity at alkaline pH in the absence of Ca^{++} can be obtained from filamentous fungi of the genera *Gliocladium Verticillium* and *Trichophaea* and that the lipolytic enzymes are effective for improving the effect of detergents. The lipolytic enzymes have a good washing performance, as expressed by the hydrolysis of oil on textile swatches. The amino acid sequences of the lipolytic enzymes are highly homologous.

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ALKALINE LIPOLYTIC ENZYME

TECHNICAL FIELD

This invention relates to an alkaline lipolytic enzyme, a detergent composition comprising the enzyme, methods of producing the enzyme, an isolated DNA sequence encoding the enzyme, a recombinant expression vector comprising the DNA sequence and cells comprising the DNA sequence or the vector,

BACKGROUND ART

For a number of years lipolytic enzymes have been used as detergent additives to remove lipid or fatty stains.

Thus, the prior art suggests the use of various lipolytic enzymes with lipase or cutinase activity as detergent additives. Examples include microbial lipolytic enzymes derived from strains of *Fusarium*, e.g. *F. oxysporum* (EP 130 064) and *F. solani f. sp. pisi* (WO 90/09446), *Humicola lanuginosa* (also called *Thermomyces lanuginosus*, EP 258 068 and EP 305 216), *Pseudomonas*, e.g. *P. alcaligenes* and *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. mendocina* (WO 88/09367), and *Bacillus*, e.g. *B. subtilis* (Dartois et al., (1993) Biochemica et Biophysica acta 1131, 253-260), *B. stearothermophilus* (JP 64/74992) and *B. pumilus* (WO 91/16422).

It is the object of this invention to provide lipolytic enzymes having good washing performance and stability in a detergent solution.

STATEMENT OF THE INVENTION

Surprisingly, we have found that alkaline lipolytic enzymes can be obtained from filamentous fungi of the genera *Gliocladium*, *Verticillium* and *Trichophaea* and that the lipolytic enzymes are effective for improving the effect of detergents. The lipolytic enzymes have a good washing performance and stability in a detergent solution.

Full length cDNA sequences each encoding a lipolytic enzyme according to the invention were derived from three strains of *Gliocladium sp.*, *Verticillium sp.* and *Trichophaea saccata* as donor organisms. The cDNA sequences were cloned into the plasmid pYES 2.0 present in *Escherichia coli.*, and the cloned *E. coli* strains were deposited by the inventors, as shown in the table below. The lipolytic enzyme encoding DNA sequence harbored in the deposited *E. coli* strain is believed to have the

sequence shown in the positions and listing indicated below, and the amino acid sequence deduced therefrom is shown in the indicated positions and listing.

The information is summarized below:

Donor organism	*Gliocladium sp.*	*Verticillium sp.*	*T. saccata*
Donor strain	CBS 173.96	CBS 830.95	CBS 804.70
E. coli transformant	DSM 10591	DSM 10590	DSM 11298
DNA sequence listing	SEQ ID NO: 2	SEQ ID NO: 5	SEQ ID NO: 7
DNA positions	114-713	133-738	161-763
Amino acid sequence listing	SEQ ID NO: 3	SEQ ID NO: 6	SEQ ID NO: 8
Amino acid positions	1-200	1-202	1-201

Homologies of the above DNA and amino acid sequences were calculated by methods described later in this specification. The following homologies were found between pairs of sequences, amino acid homology at the upper right corner, and DNA homology at the lower left. (given as DNA homology / amino acid homology):

	Gliocladium sp.	*Verticillium sp.*	*T. saccata*
Gliocladium sp.	100	91	96
Verticillium sp.	83	100	89
T. saccata	92	83	100

Accordingly, the invention in its various aspects provides:

1. A lipolytic enzyme which is:

a) a polypeptide encoded by the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 10591, DSM 10590 or DSM 11298, or

b) a polypeptide produced by *Gliocladium sp.* CBS 173.96, *Verticillium sp.* CBS 830.95 or *Trichophaea* saccata CBS 804.70, or

c) a polypeptide having an amino acid sequence as shown in positions 1-200 of SEQ ID NO: 3, positions 1-202 of SEQ ID NO: 6, or positions 1-201 of SEQ ID NO: 8, or

d) an analogue of the polypeptide defined in (a), (b) or (c) which:

i) is at least 60% homologous with said polypeptide, or

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form.

2. An alkaline lipolytic enzyme which is derivable from a strain of *Gliocladium* and has a lipolytic activity at pH 10 in the absence of Ca^{++} above 20% of the lipolytic activity at pH 10 in the presence of 50 mM Ca^{++}.

3. An alkaline lipolytic enzyme which is derivable from a strain of *Gliocladium* and gives a degree of hydrolysis above 15% on cotton/olive oil swatches in the Activity-in-Detergent (AiD) assay.

4. An alkaline lipolytic enzyme which is derivable from a strain of the genus *Verticillium* and retains more than 90% activity after 30 minutes incubation at pH 10.2, 40°C in a solution of 0.300 g/l C_{14}-C_{16} alkyl sulfate, 0.650 g/l alcohol ethoxylate (C_{12}-C_{14}, 6 EO), 1.750 zeolite P, 0.145 g/l Na_2CO_3, 0.020 g/l acrylate/maleate copolymer and 0.050 g/l carboxymethyl cellulose.

5. An enzymatic detergent composition comprising a surfactant and the lipolytic enzyme of any preceding claim.

6. A method of producing an alkaline lipolytic enzyme, comprising cultivation of a lipolytic enzyme-producing strain of *Gliocladium*, *Verticillium* or *Trichophaea* in a suitable nutrient medium, followed by recovery of the alkaline lipolytic enzyme.

7. A method for producing an alkaline lipolytic enzyme, comprising:

a) isolating a DNA sequence encoding the lipolytic enzyme from a lipolytic enzyme-producing strain of *Gliocladium*, *Verticillium* or *Trichophaea*,

b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,

c) transforming a suitable heterologous host organism with the vector,

d) cultivating the transformed host organism under conditions leading to expression of the lipolytic enzyme, and

e) recovering the lipolytic enzyme from the culture medium.

8. An isolated DNA sequence which encodes the lipolytic enzyme of any of claims 1-7.

9. An isolated, lipolytic enzyme encoding DNA sequence which comprises:

a) the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 10591, DSM 10590 or DSM 11298, or

b) the DNA sequence shown in positions 114-713 of SEQ ID NO: 2, positions 133-738 of SEQ ID NO: 5 or positions 161-763 of SEQ ID NO: 7, or

c) an analogue of the DNA sequence defined in a) or b) which

i) is at least 60% homologous with said DNA sequence, or

ii) hybridizes with said DNA sequence at 55°C.

10. A recombinant expression vector comprising the DNA sequence of any of claims 19-24.

11. A cell comprising the DNA sequence of any of claims 19-24 or the recombinant expression vector of claim 25.

12. A method of producing a lipolytic enzyme, comprising culturing the cell of any of claims 26-29 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

13. A biologically pure culture of a microbial strain which belongs to the genus *Gliocladium* or *Verticillium* and is capable of producing an alkaline lipolytic enzyme.

14. *Escherichia coli* strain DSM 10591, DSM 10590 or DSM 11298 or a mutant thereof having lipolytic enzyme encoding capability.

Comparison with prior art

Tilburg and Thomas, Application. Environ. Microbiol., Jan. 1993, p. 236-242 describes production of lipase by *G. virens*; however, data in the article show that the prior-art lipase is not alkaline. US 4,985,365 and US 4,511,655 describe the use of culture broth of *G. roseum* IFO 5422 and *G. virens* IFO 6355 to hydrolyze carboxylic esters at acid pH. The prior art does not describe the production of lipolytic activity at alkaline pH by strains of *Gliocladium*.

The prior art describes the production of lipase by *Verticillium cinnabarinum* (also called *V. luteoalbum*) DSM 63078 (Rapp & Backhaus, Enzyme Microb. Technol., 14, 938-943 (1992)) and *Verticillium lecanii* ATCC 26854 (JP-A 61-289884). The

inventors have investigated the two strains and found that they do not produce alkaline lipolytic enzyme.

The following literature describes lipase production by the genus *Verticillium* without identifying any particular strains: Kunert & Lysek, Biologica (Bratislava), 42 (3), 285-293 (1987). Leger et al., J. Invertebr. Pathol., 48, 85-95 (1986). Jackson et al., Ann. appl. Biol., 106, 39-48 (1985). Roberts et al., Mycologia, 79 (2), 265-273 (1987). Trigiano, Mycologia, 71, 908-917 (1979). However, the prior art does not describe the production of lipolytic activity at alkaline pH by strains of *Verticillium*.

A homology search was performed in nucleotide and protein databases. The highest homology for the lipolytic enzyme and DNA sequences of the invention was found with the sequence for cutinase from *Fusarium solani f. sp. pisi*, described by C.L. Soliday et al., Proc. Natl. Acad. Sci. USA, 81, 3939-3943 (1984).

The three DNA sequences of the invention described earlier in this specification show homologies of 53-57% with the above known DNA sequence, and the three amino acid sequences of the invention described earlier show homologies of 50-53% with the above known amino acid sequence. The calculation of homology was done as described later in this specification. Using a formula given in "Current Protocols in Molecular Biology", John Wiley & Sons, 1995, hybridization of the above DNA of the invention and the closest prior-art DNA is estimated to have a melting temperature of 50°C at the hybridization conditions given later in this specification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1-7 show pH-activity curves for lipolytic enzymes from the following strains. The pH curves were made with purified enzyme samples, except that those in Figs. 3-5 were made with crude enzyme samples.

Fig. 1: *Gliocladium sp.* NN140631

Fig. 2: *G. solani* NN102998

Fig. 3: *G. roseum* NN141784

Fig. 4: *G. aureum* NN102987

Fig. 5: *G. roseum* NN141961

Fig. 6: *Verticillium sp.* CBS 830.95

Fig. 7: *T. saccata* CBS 804.70

Fig. 8 shows the stability at various temperatures for the lipolytic enzyme from *Verticillium sp.* CBS 830.95.

DETAILED DISCLOSURE OF THE INVENTION

Lipolytic enzymes

The enzymes of this invention are lipolytic enzymes. In the present context the term "lipolytic enzyme" is intended to indicate an enzyme classified under the Enzyme Classification number E.C. 3.1.1.- (Carboxylic Ester Hydrolases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB). Lipolytic enzymes thus exhibit hydrolytic activity towards at least one of the types of ester bonds mentioned in the context of E.C. 3.1.1.

The lipolytic enzymes of the invention preferably have lipase activity (with triglycerides as substrate) and/or cutinase activity (with cutin as substrate, as described in Kolattukudy, Science, vol. 208, 30 May 1980, pp. 990-1000 and Kolattukudy in "Lipases", Borgström and Brockman ed., Elsevier 1984, pp. 471-504).

Properties of lipolytic enzyme

The invention provides lipolytic enzymes having a high activity at alkaline pH in the absence of Ca^{++}. Preferably, the alkaline lipolytic enzyme of the invention has a lipolytic activity at pH 10 in the absence of Ca^{++} above 20% (most preferably above 50%) of the lipolytic activity at pH 10 in the presence of 50 mM Ca^{++}. And preferably, the lipolytic enzymes have a lipolytic activity at pH 10 in the absence of Ca^{++} above 50% of the activity at pH 8 as well as pH 9 in the absence of Ca^{++}. Such an enzyme can be obtained from a strain of *Gliocladium*.

Curves of lipolytic activity versus pH with and without addition of Ca^{++} are shown in Figs. 1-7 for lipolytic enzymes according to the invention from the following strains: *Gliocladium sp.* NN140631, *G. solani* NN102998, *G. roseum* NN141784, *G. aureum* NN102987, *G. roseum* NN141961, *Verticillium sp.* CBS 830.95 and *T. saccata* CBS 804.70. The activity was determined by the OPID method described later in this specification (except that 60 minutes incubation was used for the data in Fig. 4). The pH curves were made with purified enzyme samples, except that those in Figs. 3-5 were made with crude enzyme samples.

Advantageously, the lipolytic enzymes of the invention are active throughout the pH range 8-10. Some preferred enzymes have increasing activity up to pH 10, indicating a pH optimum above 10.

The specific lipolytic enzyme activity is 1800 LU per A_{280} for the lipolytic enzyme from *Verticillium sp.* CBS 830.95 The specific activity is expressed as lipase activity (LU) per mg of protein determined from absorption at 280 nm.

The stability is shown in Fig. 8, as expressed by the residual activity after incubating the lipolytic enzyme from *Verticillium sp.* CBS 830.95 at various temperatures for 30 minutes at pH 9. The enzyme is fully stable for 30 minutes at pH 9 at temperatures up to 50°C. This enzyme was also found to be fully stable throughout the pH range 6-10 at 25°C for 24 hours.

The invention also provides lipolytic enzymes having a high stability in a detergent solution. Preferably, the alkaline lipolytic enzyme of the invention retains more than 90% activity after 30 minutes incubation in 100 mM glycine at pH 10, 45°C or in the test detergent solution shown in the Examples at pH 10.2, 40°C. The lipolytic enzymes of the enzymes furthermore show a good washing performance on fatty soiling during the washing of textiles with detergent. Preferably, the alkaline lipolytic enzyme of the invention gives a degree of hydrolysis above 15% (most preferably above 20%) on cotton/olive oil swatches in the Activity-in-Detergent (AiD) assay described later in this specification. Such an enzyme can be obtained from a strain of *Verticillium*.

In this specification, lipolytic enzyme activity is expressed in units of LU, OPIDU and SLU determined by the methods described below.

Characterization of enzyme protein

The iso-electric point was determined by iso-electric focusing for some lipolytic enzymes according to the invention, as follows:

Organism	Strain No.	Iso-electric point
G. solani	NN102998	8.4
Gliocladium sp.	NN140631	9.3
Verticillium sp.	CBS 830.95	6.0

The molecular weight (MW) was determined by SDS-PAGE and by mass spectrometry for some lipolytic enzymes according to the invention, as follows:

Organism	Strain No.	MW (SDS-PAGE)	MW (mass spectrometry)
G. solani	NN102998	22 kDa	20,989 ± 21 Da
Verticillium sp.	CBS 830.95	22 kDa	21,107± 21 Da

The N-terminal sequence of the lipolytic enzyme from *G. solani* NN102998 was determined for 35 residues as shown in SEQ ID NO: 1. The complete amino acid sequence of the lipolytic enzyme from *Gliocladium sp.* CBS 173.96 was deduced from the determination of the DNA sequence and is shown in positions 1-200 of SEQ ID

NO: 3. A comparison of the two amino acid sequences shows that the first 35 amino acids of the two enzymes are identical, except for position 20.

The N-terminal sequence determined for the lipolytic enzyme from *Verticillium sp.* CBS 830.95 is shown in SEQ ID NO: 4 (positions 1-29); Xaa indicates an undetermined amino acid. The complete amino acid sequence of this enzyme, as deduced from the DNA sequence, is shown in positions 1-202 of SEQ ID NO: 6.

The amino acid sequence of the lipolytic enzyme from *T. saccata* CBS 804.70 shown in positions 1-201 of SEQ ID NO: 8 was deduced from the DNA sequence, and the position of the N-terminal was deduced by a comparison with the highly homologous sequence from *Gliocladium sp.* CBS 173.96.

Lipolytic Activity by the LU Method

One Lipase Unit (LU) is the amount of enzyme which liberates 1 μmol of titratable fatty acid per minute with tributyrin as substrate and gum arabic as emulsifier at 30.0°C, pH 7.0 (phosphate buffer).

Lipase Activity by the OPID Method

The lipolytic enzyme activity without free Ca^{++} in the range pH 7-10 is tested with a substrate emulsion of olive oil: 2% PVA solution (1:3)at 40°C for 10 minutes, at a specified pH. At the end of the reaction, the reaction mixture is extracted by chloroform: methanol (1:1) at acidic conditions, and the fatty acid released during the reaction is measured by TLC-FID analysis (Iatroscan). One unit (OPIDU) is taken as the release of a μmole of fatty acid per minute.

In each test, 10 mM EDTA is used together with 200 mM of buffer (Tris-HCl buffer at pH 7 and 8, diethanol amine buffer at pH 8, 9 and 10).

Lipolytic Activity by the SLU Method

The lipolytic activity may be determined using olive oil as substrate. In this SLU method, the lipase activity is measured at 30°C and pH 9 with a stabilized olive oil emulsion (Sigma catalog No. 800-1) as the substrate, in a 5 mM Tris buffer containing 40 mM NaCl and 5 mM calcium chloride. 2.5 ml of the substrate is mixed with 12.5 ml buffer, the pH is adjusted to 9, 0.5 ml of diluted lipase sample is added, and the amount of oleic acid formed is followed by titration with a pH stat.

One SLU is the amount of lipase which liberates 1 μmole of titratable oleic acid per minute under these conditions.

Activity-in-Detergent (AiD) assay

Equipment: Water bath with 150 ml beakers. Stirring is obtained by an agitator.

Lipolytic enzyme dosage: 0 & 12500 LU/l.

Substrate: 6 pieces (3.5*3.5 cm) of cotton with 6 µl olive oil

Detergent: 0.5 g/l model liquid detergent (see below) dissolved in 0,36 mM Ca^{2+}/Mg^{2+} (5:1), adjusted to pH 10. 100 ml per beaker.

Method: The test swatches are added to the detergent solution, after which the samples get stirred for 60 min at 30°C. The remaining detergent on the swatches gets removed by rinsing in tap water for 15 min. The swatches are put into a flask containing 10 ml tetrahydrofuran and 6.25 µl 4 M HCl and evaporated over night, after which the samples are redissolved in tetrahydrofuran. The effect of the lipolytic enzyme is determined:

By measuring the degree of hydrolysis (% DH) by an Iatroscan TLC/FID method

Model liquid detergent:

Component	Model detergent, % w/w
Linear alkylbenzene sulfate (LAS)	17.5
Alcohol ethoxylate (AEO)	14.4
Dodecenyl/tetradecenyl succinic acid (DTSA)	10
Oleic acid	3
Coconut oil	5
Mono ethanol amine (MEA)	14.5
Mono propylene glycol (MPG)	10.7
Ethanol	1,4
Phosphonate	1.0
Boric acid	0.8
Citric acid	3.9
Sodium chloride	0.13
Potassium chloride	0.38
Hydrochloric acid 4 M	6
Water	9.7
pH adjusted to (5 g/l)	7.7

Microbial sources

The lipolytic enzyme of this invention may be derived from an ascomycete of the order *Hypocreales* which belongs to the genus *Gliocladium*, *Verticillium* or *Trichophaea*.

The genus *Gliocladium* is characterized by having one-celled conidia formed from phialides in slimy heads. The conidiophores are distinctly penicillate. It is described in Domsch K.H. & Gams W. (1993) Compendium of Soil Fungi (reprint of 1980 edition), Volume I, IHW-Verlag, page 368.

The genus *Verticillium* is characterized by predominantly hyaline hyphae with well differentiated erect conidiophores that are verticillately branched. The branches bear whorls of slender phialides from which hyaline or brightly colored conidia are formed. The conidial masses are seen as slimy heads on top of the phialidia.

The following species and strains are preferred. Variants and mutants thereof capable of producing lipolytic enzyme may also be used in the invention.

Species name	Inventors' strain No.	Deposit number	Deposit date
Gliocladium sp.	NN140631	CBS 173.96	February 5,1996
G. ammoniophilum	NN102992	CBS 156.70	
G. aureum	NN102987	IFO 9055	
G. catenulatum	NN100802	NRRL 1091	
G. flavum	NN102995	CBS 155.27	
G. nigrovirens	NN102996	CBS 183.30	
G. roseum	NN141784	CBS 126.96	January 22, 1996
	NN141961	CBS 127.96	January 22, 1996
G. sagariensis	NN102989	IFO 9080	
G. solani	NN102998	CBS 707.86	
Verticillium sp.	NN001755	CBS 830.95	22 December 1995
T. saccata	NN102806	CBS 804.70	

The deposit numbers in the above list refer to deposits made at the following deposit institutions:

CBS: Centraal Bureau voor Schimmelcultures, Oosterstraat 1, 3740 AG Baarn, Netherlands.

IFO: Institute for Fermentation, 17-85 Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan.

NRRL: Agricultural Research Service Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604, USA.

The following strains were isolated by the inventors: *Gliocladium sp.* CBS 173.96, *G. roseum* CBS 126.96, *G. roseum* CBS 127.96 and *Verticillium sp.* CBS 830.95. These strains were deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the deposit numbers and dates given in the table above. They were classified by standard taxonomic methods. Two strains are denoted as "sp.", indicating that they could not be identified to species level. *Verticillium sp.* CBS 830.95 was isolated from leaf-material and thus most likely belongs to the group of saprophytic species on plant material.

Transformant *E. coli* strains

Expression plasmids comprising the full length cDNA sequence encoding lipolytic enzymes of the invention from three of the above strains were transformed into strains of *Escherichia coli* as indicated earlier in this specification. The transformants were deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, (DSM). The deposit numbers and dates of the transformed *E. coli* strains were as follows:

Deposit number	Deposit date
DSM 10591	15 March 1996
DSM 10590	15 March 1996
DSM 11298	27 November 1996

DNA sequence

In this specification and claims, whenever reference is made to the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in a transformed *E. coli* strain, such reference is also intended to include the lipolytic enzyme encoding part of the corresponding DNA sequence listing as identified earlier in this specification. Accordingly, the terms may be used interchangeably.

The DNA sequence of the invention may be isolated from the deposited transformant of *Escherichia coli* by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

The DNA sequence of the invention may also be isolated from a strain of the genus *Gliocladium*, *Verticillium* or *Trichophaea* producing the lipolytic enzyme of the invention or another or related organism and thus, e.g. be an allelic or species variant of the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in a transformant of *Escherichia coli* identified earlier in this specification.

Alternatively, the sequence may be constructed on the basis of the DNA sequence presented as the lipolytic enzyme encoding part of the indicated sequence listings, e.g., it may be a sub-sequence thereof, and/or be derived by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the lipolytic enzyme encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see e.g. Ford et al., (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. e.g. Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. lipolytic enzyme) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as

nuclear magnetic resonance analysis, crystallography or photoaffinity labeling (cf. e.g. de Vos et al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

The DNA sequence of the invention can be isolated from the transformed *E. coli* strain by extraction of DNA by methods known in the art, e.g. as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be isolated by any general method involving

-cloning, in suitable vectors, a cDNA library from any organism expected to produce the lipolytic enzyme of interest,

-transforming suitable yeast host cells with said vectors,

-culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,

-screening for positive clones by determining any lipolytic enzyme activity of the enzyme produced by such clones, and

-isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 or WO 94/14953, the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in the Examples below.

Alternatively, the DNA encoding a lipolytic enzyme of the invention may, in accordance with well-known procedures, conveniently be isolated from a suitable source, such as the microorganisms described above, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the lipolytic enzyme encoding part of the nucleotide sequences presented as SEQ ID NO: 2 or any suitable subsequence thereof, or on the basis of the amino acid sequence SEQ ID NO: 3.

Homology of DNA sequences

The DNA sequence homology referred to in this specification with claims is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48,

443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous DNA sequences referred to above exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the lipolytic enzyme encoding part of the DNA sequence indicated earlier in the specification.

Hybridization

The hybridization referred to above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the lipolytic enzyme under certain specified conditions which are described in detail below. The oligonucleotide probe to be used is the DNA sequence corresponding to the lipolytic enzyme encoding part of the DNA sequence listings indicated earlier in the specification.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5% SDS at temperatures up to 55°C, preferably up to 60°C, more preferably up to 65°C, even more preferably up to 70°C, and especially up to 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

Homology of amino acid sequences

The polypeptide homology referred to in this specification with claims is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for polypeptide sequence comparison:

GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature part of a polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and especially at least 97% with the mature part of the amino acid sequence of lipolytic enzymes indicated earlier in this specification.

Immunological cross-reactivity:

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified lipolytic enzyme. More specifically, antiserum against the lipolytic enzyme of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ((NH4)2 SO4), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Expression vectors

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the lipolytic enzyme should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the lipolytic enzyme, the promoter and the terminator, respectively, and to

insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α-amylase, *Aspergillus niger* acid stable α-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host cells

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell. Preferred filamentous fungi include *Aspergillus*, *Fusarium* or *Trichoderma*, most preferably *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis*, *T. harzianum* or *T. reesei*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* such as *Schizosaccharomyces pombe*, a strain of *Hansenula*, *Pichi*, *Yarrowia* (such as *Yarrowia lipolytica)* or *Kluyveromyces* (such as *Kluyveromyces lactis)*.

Production of lipolytic enzyme

The lipolytic enzyme of the invention may be produced by cultivation of one of the microorganisms described above in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the lipolytic enzyme. An alternative method of producing the lipolytic enzyme of the invention comprises transforming a suitable host cell with a DNA sequence encoding the enzyme, cultivating the transformed organism under conditions permitting the production of the enzyme, recovering the enzyme from the culture.

The medium used to culture the microorganism or transformed host cells may be any conventional medium suitable for growing the organism in question. The expressed lipolytic enzyme may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Application of lipolytic enzyme

The lipolytic enzyme of the invention may be used in conventional applications of lipolytic enzyme, particularly at a high pH, e.g. in laundry and dishwash detergents, in institutional and industrial cleaning and in leather processing.

The lipolytic enzymes of the invention can also be used for interesterification, for total hydrolysis of fats and oils and in optical isomer resolution processes.

Detergent additive

According to the invention, the lipolytic enzyme may typically be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme.

A suitable activity range for a detergent additive containing the lipolytic enzyme of this invention is 5,000-100,000 OPIDU/g (OPID measured at pH 9) or 0.01-100 mg pure enzyme protein per g of the additive.

Detergent

The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. The detergent composition of the invention may comprise lipolytic enzyme in an amount corresponding to 10-50,000 LU per gram of detergent, preferably 20-5,000 LU/g. The detergent may be dissolved in water to produce a wash liquor containing lipolytic enzyme in an amount corresponding to 25-15,000 LU per liter of wash liquor. The amount of lipolytic enzyme protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

Detergent Compositions

According to the invention, the lipolytic enzyme may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-

dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethylene glycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene sulfonate (LAS), alpha-olefin sulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkane sulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as amylase, cutinase, protease, cellulase, peroxidase, and oxidase, e.g., laccase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethyl cellulose (CMC), poly(vinyl pyrrolidone) (PVP), polyethylene glycol

(PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzene sulfonate (NOBS). Alternatively, the bleaching system may comprise peroxy acids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaAlSiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaAlSiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaAlSiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaAlSiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

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8) A detergent composition formulated as a granulate comprising

Linear alkylbenzene sulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%

Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzene sulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaAlSiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_8H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluene sulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethyl cellulose	0 - 1%

Linear alkylbenzene sulfonate (calculated as acid)	15 - 23%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzene sulfonate, alkyl sulfate, alpha-olefin sulfonate, alpha-sulfo fatty acid methyl esters, alkane sulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaAlSiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzene sulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaAlSiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipolytic enzyme may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of lipolytic enzyme per liter of wash liquor.

EXAMPLES

Materials And Methods

The following materials and methods were used in the Examples that follow:

Microorganisms:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATα; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

E. coli strain: DH10B (available, e.g., from Life Technologies)

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (available, e.g., from Invitrogen)

General molecular biology methods:

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Expression cloning in yeast

Expression cloning in yeast was done as comprehensively described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference.

Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries were done according to the references mentioned above.

Identification of positive clones:

The transformants are plated on SC agar containing 2% glucose and incubated for 3 days at 30°C. A cellulose acetate filter (OE 67, Schleicher & Schuell) is placed on top of the cells and then transferred to plates containing SC agar and 2% galactose with the cells on the top of the filter. After 3 days of incubation at 30°C the filter with cells is transferred to substrate plates. Positive clones are identified as colonies surrounded by a green zone.

Characterization of positive clones:

The positive clones are obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (Sanger et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467) and the Sequenase system (United States Biochemical).

Isolation of a cDNA gene for expression in *Aspergillus*:

A lipolytic enzyme-producing yeast colony is inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube is shaken for 2 days at 30°C. The cells are harvested by centrifugation for 10 min. at 3000 rpm.

DNA is isolated according to WO 94/14953 and dissolved in 50 µl water. The DNA is transformed into *E. coli* by standard procedures. Plasmid DNA is isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The

cDNA insert is excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.

Transformation of Aspergillus oryzae or Aspergillus niger

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants is inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed. The lipolytic activity is identified by applying 10 µl supernatant to 4 mm diameter holes punched out in agar plates containing 0.1 M glycine pH 9, 0.1 M $CaCl_2$, 1% Triton X-100, 0.5% olive oil. Lipolytic activity is indicated by the formation of a turbid halo.

Fed batch fermentation:

Fed batch fermentation was performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H_2O ad 900 ml, autoclaved

Substrate plates: Petri dish containing 100 mM glycine, pH 9.0, 1% brilliant green solution, 2.5 mM $CaCl_2$, 0.6% olive oil, 0.036% polyvinyl alcohol (MW 70,000-100,000, Sigma P-1763)

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

Ingredient	Composition of medium (g/l)					
	Agar30	YS-2	Gli	MT-C	NOMO 16	YS-25
Peptone	6	10	10	5	6	10
Pepticase	4				4	
Soybean powder				30		
Corn steep powder				5		
Yeast extract	3	10		1	3	10
Meat extract	1.5				1.5	
Glucose	1	20		10	1	5
NH_4NO_3				2.5		
K_2HPO_4		5	5	4		5
$MgSO_4 \cdot 7H_2O$		1	1	0.1		1
Olive oil	20		20			
Corn oil				10 or 20		
Soybean oil						20
Sorbitan monostearate					20	
pH adjusted to	7.4	6.5	7.0	7.0	7.4	6.5

Example 1

Lipase production from strains of *Gliocladium* and *Trichophaea*

Each of the *Gliocladium* strains shown in the table below was used for lipolytic enzyme production by a seed culture followed by a main culture. The seed culture was made by cultivation on YS-2 medium for 2 days at 27°C, and the main culture was made at 27°C using the medium and culture time shown below. At the end of the main culture, the cells were removed and the yield of lipolytic activity was measured using the LU and SLU assay methods.

Species	Strain	Main culture		Lipase activity	
		Medium	Days	LU/ml	SLU/ml
G. catenulatum	NN100802	MT-O	5	2.3	-
G. aureum	NN102987	Agar30	3	2.4	1.7
G. sagariensis	NN102989	MT-C	3	0.7	1.2
G. ammoniophilum	NN102992	Agar30	3	3.0	2.7
G. flavum	NN102995	Gli	3	2.1	1.5
G. nigrovirens	NN102996	Gli	3	3.2	0.9
G. solani	NN102998	Agar30	5	36.0	-
Gliocladium sp.	NN140631	Agar30	3	4.4	-
G. roseum	NN141784	Agar30	5	2.6	-
G. roseum	NN141961	MR-10	3	9.0	-
T. saccata	NN102806	YS-25	3	5.2	-

All the above strains were seen to produce lipolytic enzyme. A particularly high yield was found by cultivation of *G. solani.*

Example 2

Activity of lipolytic enzymes from *Gliocladium* and *Trichophaea* at various pH

The cell-free culture broths from Example 1 were tested for lipolytic enzyme activity at pH 6.0, 8.5 and 10.0 without the addition of Ca^{++} and at pH 10 with addition of Ca^{++}. The plate test described in Example 11 of WO 88/02775 (corresponding to JP-W 1-501120) was used.

Species	Strain	Lipase activity			
		pH 6	pH 8.5	pH 10	pH 10 + Ca^{++}
G. catenulatum	NN100802	-	2	2	2
G. aureum	NN102987	1	1	1	1
G. sagariensis	NN102989	1	2	2	2
G. ammoniophilum	NN102992	1	2	2	2
G. flavum	NN102995	2	3	2	2
G. nigrovirens	NN102996	1	1	1	1
G. solani	NN102998	2	2	3	3
Gliocladium sp.	NN140631	-	2	2	-
G. roseum	NN141784	-	2	2	-
G. roseum	NN141961	-	1	1	-
T. saccata	NN102806	-	2	2	-

It is seen that in this semi-quantitative test, all the above lipase preparations show nearly the same activity in the range pH 6-10, with and without calcium addition.

Example 3

Production of lipolytic enzyme from *Gliocladium sp.*

A seed culture was prepared by inoculating *Gliocladium sp.* CBS 173.96 from a slant of PDA (product of Difco) to one shake flask with shaking for 2 days at 27°C. A main culture was prepared by using the seed culture to inoculate 50 shake flasks with 100 ml of NOMO 16 for 5 days at 27°C with shaking.

3,000 ml of cell-free broth with a lipase activity of 11 LU/ml was recovered after removal of the cell mass, that was directly employed for the purification.

Example 4

Purification of lipolytic enzyme from *Gliocladium sp.*

0.5% CHAPS was added to the culture broth from Example 3. This was centrifuged at 45,000 rpm for 1 hour and filtered on a 0.8 µm filter. The filtrate was applied onto a gel filtration column (Superdex, product of Pharmacia) using 50 mM Tris-HCl buffer (pH 8.5 with 0.2 M NaCl.

Example 5

Production of lipolytic enzyme from *G. solani*

A seed culture was prepared by inoculating G. solani CBS 707.86 from a slant of PDA (product of Difco) to two shake flasks with shaking for 2 days at 27°C. A main culture was prepared by using the seed culture to inoculate 50 shake flasks with 100 ml of Agar 30 for 5 days at 27°C with shaking. 4,900 ml of cell-free broth with a lipase activity of 49 LU/ml was recovered after removal of the cell mass. This was deionized and free-dried to obtain 10.2 g of powder sample with a lipase activity of 15,700 LU/g.

Example 6

Purification of lipolytic enzyme from *G. solani*

The lipolytic enzyme was purified by 2 steps, hydrophobic interaction and gel filtration. More specifically, the purification was performed as follows.

The powder sample from Example 5 was dissolved in 3 M ammonium acetate including 0.5% 3[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and centrifuged at 18,000 rpm for 20 minutes. The supernatant was filtered with 0.2 µm filter and applied onto Butyl Toyopearl column chromatography (62 x 200 mm). After unbound materials were washed out by 3 M ammonium acetate and then the column was washed by 50 mM sodium carbonate buffer (pH 10.0) including 0.5% CHAPS. Lipolytic activity was eluted by H_2O. The eluted lipolytic enzyme was applied onto gel filtration column (26 x 600 mm). The applied volume was 3 ml and the eluent was 35 mM sodium carbonate buffer (pH 10.0) including 0.3% CHAPS. The flow rate was 3 ml/min. The lipolytic enzyme was eluted around 225 ml.

A molecular weight of 36 kDa was calculated from the gel filtration.

Fractions containing lipolytic activity were pooled and dialyzed/concentrated by ultra-filtration. A molecular weight of the lipolytic enzyme of 22 kD was calculated form SDS-PAGE. An iso-electric point between 8.15 and 8.45 was found by IEF-PAGE.

Example 7

Production of lipolytic enzyme from *Verticillium sp.*

Seed cultures of *Verticillium sp.* CBS 830.95 were produced in 500 ml shake flasks containing 150 ml medium of the following composition:

Corn steep liquor: 12 g/l

Glucose: 24 g/l

To each flask is added 0.5 ml of oil and 0.5 g of $CaCO_3$.

pH is adjusted to 5.5 before autoclavation.

The flasks were inoculated with spore suspensions from slants, using 10 ml per shake flask.

After 2 days at 26°C at 200 rpm, the seed culture was used for inoculation of shake flasks containing 150 ml of the following medium:

Peptone	6 g/l
Pepticase	4 g/l
Yeast extract	3 g/l
Beef extract	1.5 g/l
Dextrose	1 g/l
Olive oil	10 g/l

pH is adjusted to 7.3-7.4 before autoclavation

Each flask was inoculated with 4 ml seed culture. The flasks were incubated at 26°C at 200 rpm for 4 days.

Two flasks yielded respectively 6.3 LU/ml and 7.6 LU/ml.

50 flasks resulted in 4.7 l of broth which was purified.

Example 8

Purification of lipolytic enzyme from *Verticillium sp.*

4 l of culture broth obtained as in Example 1 with an activity of 4.4 LU/ml was purified by the following procedure.

Decyl Agarose (50 ml): The culture broth was filtered and applied on a Decyl Agarose column previously equilibrated in 10 mM Tris/0.25 M NaCl, pH 7. Bound proteins were eluted with 50% ethanol. Yield: 75%.

Q Sepharose (25 ml): The Decyl Agarose fraction was applied on a Q Sepharose column previously equilibrated in 10 mM H_3BO_3/KCl, pH 10 after adjusting pH to 10. Activity was eluted from 0-0.25 M NaCl using a linear gradient. Yield: 50%.

Concentration: Desalting was carried out on G-25, followed by speed vacuum freeze drying. Yield: 60%.

Example 9

Wash performance of lipolytic enzyme

Lipolytic enzyme of the invention was compared to prior-art enzymes in the following washing test:

A lipolytic enzyme according to this invention (from *G. solani* NN102998) was tested by the above AiD assay and compared to a prior-art enzyme: Lipolase® (a lipase derived from *Humicola lanuginosa*).

	Lipase	% hydrolysis on olive oil
Invention	*G. solani* NN102998	23
Prior art	Lipolase	8
Blank	None	1

It is seen that the wash effect of the lipase of this invention is far superior to the prior art.

Example 10

Stability of lipolytic enzyme in detergent solution

The purified lipolytic enzyme from Example 2 was incubated for 30 minutes in each of the solutions shown below. The lipase activity was measured before and after the incubation, and the stability was expressed as % residual activity. Results:

100 mM glycine, pH 10, 45°C 97%

Test detergent (see below), pH 10.2, 40°C 99%

The above results demonstrate an excellent stability at alkaline pH, even in the presence of detergent.

The test detergent solution had the following composition (in g/l):

Alkyl sulfate (C_{14}-C_{16})	0.300
Alcohol ethoxylate (C_{12}-C_{14}, 6 EO)	0.650
Zeolite P	1.750
Na_2CO_3	0.145
Acrylate/maleate copolymer	0.020
Carboxymethyl cellulose	0.050

Example 11

Cloning and expression of lipolytic enzymes

In this example, lipolytic enzymes from *Gliocladium sp.* CBS 173, *Verticillium sp.* CBS 830.95 and *T. saccata* CBS 804.70 as donor organisms were cloned and expressed, using the method for "Expression cloning in yeast" described previosuly in this specificaiton.

mRNA was isolated from the donor organism, cultivated essentially as in the main culture of a preceding example with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library therefrom, consisting of approx. 9×10^5 individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA

from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Lipolytic enzyme-positive colonies were identified and isolated as described above. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the lipolytic enzyme was determined. The DNA sequence, the corresponding amino acid sequence and the lipolytic enzyme encoding region are shown in the sequence listings identified earlier in this specification.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the lipolytic enzyme in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the lipolytic enzyme gene was purified. The gene was subsequently ligated to pHD414 and digested with appropriate restriction enzymes. The resulting plasmid from each of the three donor organisms is denoted pA2L123, pA2L114 and pC1L160, respectively.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Each of the transformants were tested for lipolytic enzyme activity as described above. Some of the transformants had lipolytic enzyme activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the lipolytic enzyme in *Aspergillus oryzae*.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45-4444-8888
(H) TELEFAX: +45-4449-3256

(ii) TITLE OF INVENTION: Alkaline Lipolytic Enzyme

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: G.solani
(B) STRAIN: NN102998

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Glu Asp Ser Ile Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu Arg
1               5                  10                  15

Asn Gly Gly Gly Ala Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg Gly
            20                  25                  30

Thr Met Glu
        35
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 914 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Gliocladium sp.
(B) STRAIN: NN140631

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:21..713

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:114..713

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
TGATTTTTCA ACTCTGCATC ATG AAG TTC CTC TAC GTC GTC CAG ACC TTG          50
                       Met Lys Phe Leu Tyr Val Val Gln Thr Leu
                       -31 -30                 -25

ATC GCC CTC GCC TTG GCT AGG CCA TTG CCT GAG ACG GCT GTG GAA GTT        98
Ile Ala Leu Ala Leu Ala Arg Pro Leu Pro Glu Thr Ala Val Glu Val
    -20                 -15                 -10

GAC CTG CAG AAC CGA GAA GAT TCT ATC GGC ATA TCC TCT GTC CTT GTG       146
Asp Leu Gln Asn Arg Glu Asp Ser Ile Gly Ile Ser Ser Val Leu Val
 -5                  1               5                  10

CGT GAC GAG CTG CGC AAT GGC GGC AGC GCG TGC CCC AAG GCC ATT CTC       194
Arg Asp Glu Leu Arg Asn Gly Gly Ser Ala Cys Pro Lys Ala Ile Leu
            15                  20                  25

ATC TTT GCT CGA GGC ACA ATG GAG CTG GAT AAC ATG GGC TTA TTG GTC       242
Ile Phe Ala Arg Gly Thr Met Glu Leu Asp Asn Met Gly Leu Leu Val
        30                  35                  40

GGG CCA GCT CTT GCA GGT GGC TTA GAG GGC ATC TTG GGT TCG AAC AAC       290
Gly Pro Ala Leu Ala Gly Gly Leu Glu Gly Ile Leu Gly Ser Asn Asn
    45                  50                  55
```

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CTC TGG GTT CAA GGG GTG GGT GGC CAA TAT GCC GCC AAC CTT GAG GGC      338
Leu Trp Val Gln Gly Val Gly Gly Gln Tyr Ala Ala Asn Leu Glu Gly
 60                  65                  70                  75

AAT CTA TTT CCA GAT GGA ACA CCT CCT AAA GCC ATC CAG GAG ATG CTT      386
Asn Leu Phe Pro Asp Gly Thr Pro Pro Lys Ala Ile Gln Glu Met Leu
                 80                  85                  90

AGC CTG CTC CAA TTG GCG GAC ACC AAG TGC CCA AAC TCT AAG ATT GTT      434
Ser Leu Leu Gln Leu Ala Asp Thr Lys Cys Pro Asn Ser Lys Ile Val
             95                 100                 105

ACA GGG GGT TAT AGC CAA GGT GCT GCA CTC GTG GCC GCT GCT ATT CGC      482
Thr Gly Gly Tyr Ser Gln Gly Ala Ala Leu Val Ala Ala Ala Ile Arg
        110                 115                 120

GAT GTC AAG GCT TCC ATT CGA CAA AAG ATT GTG GGA ACC GTA CTC TTT      530
Asp Val Lys Ala Ser Ile Arg Gln Lys Ile Val Gly Thr Val Leu Phe
    125                 130                 135

GGG TAT ACT AAA AAC AAA CAG AGG AAC GGA CAG GTA GAA AAC TAC TCA      578
Gly Tyr Thr Lys Asn Lys Gln Arg Asn Gly Gln Val Glu Asn Tyr Ser
140                 145                 150                 155

ACT GAT CGG CTC CGG GTT TAC TGT AAC CTC GGA GAC TTG ATT TGT GAA      626
Thr Asp Arg Leu Arg Val Tyr Cys Asn Leu Gly Asp Leu Ile Cys Glu
                160                 165                 170

GGG ACC TTG ATT GTT CTA CCA CCA CAT CTT CTT TAT GGA GTC CAG GCT      674
Gly Thr Leu Ile Val Leu Pro Pro His Leu Leu Tyr Gly Val Gln Ala
            175                 180                 185

GCT GGT CCA GCT GCC CAG TTC CTC GCC AGC AAG ATC AAT TAATTTTTCT      723
Ala Gly Pro Ala Ala Gln Phe Leu Ala Ser Lys Ile Asn
        190                 195                 200

TGATCAATGC ATGGCAGAAT GCTGCCATGT ACTCAGATAT GGATAGGAGA GATCATATAT    783

GGACTATATA TAGTAGCTCT GCCGCATCTG TCGAAAGTTT TGATATTCTT TCGTTCGTTG    843

TTAGGGCTGA CTTATTCTTG AGATGAATAA AAAAAGATCT GTATAAAGAG AAAAAAAAAA    903

AAAAAAAAAA A                                                        914
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Met Lys Phe Leu Tyr Val Val Gln Thr Leu Ile Ala Leu Ala Leu Ala
-31 -30                 -25                 -20

Arg Pro Leu Pro Glu Thr Ala Val Glu Val Asp Leu Gln Asn Arg Glu
-15                 -10                  -5                   1

Asp Ser Ile Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu Arg Asn
              5                  10                  15

Gly Gly Ser Ala Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg Gly Thr
         20                  25                  30

Met Glu Leu Asp Asn Met Gly Leu Leu Val Gly Pro Ala Leu Ala Gly
     35                  40                  45

Gly Leu Glu Gly Ile Leu Gly Ser Asn Asn Leu Trp Val Gln Gly Val
 50                  55                  60                  65

Gly Gly Gln Tyr Ala Ala Asn Leu Glu Gly Asn Leu Phe Pro Asp Gly
                 70                  75                  80

Thr Pro Pro Lys Ala Ile Gln Glu Met Leu Ser Leu Leu Gln Leu Ala
             85                  90                  95

Asp Thr Lys Cys Pro Asn Ser Lys Ile Val Thr Gly Gly Tyr Ser Gln
        100                 105                 110

Gly Ala Ala Leu Val Ala Ala Ala Ile Arg Asp Val Lys Ala Ser Ile
    115                 120                 125

Arg Gln Lys Ile Val Gly Thr Val Leu Phe Gly Tyr Thr Lys Asn Lys
130                 135                 140                 145

Gln Arg Asn Gly Gln Val Glu Asn Tyr Ser Thr Asp Arg Leu Arg Val
                150                 155                 160

Tyr Cys Asn Leu Gly Asp Leu Ile Cys Glu Gly Thr Leu Ile Val Leu
            165                 170                 175

Pro Pro His Leu Leu Tyr Gly Val Gln Ala Ala Gly Pro Ala Ala Gln
        180                 185                 190

Phe Leu Ala Ser Lys Ile Asn
    195                 200
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Verticillium sp.
(B) STRAIN: CBS 830.95

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Glu Asp Ser Phe Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu Ile
1 5 10 15

Asn Gly Gly Gly Ala Xaa Pro Lys Ala Ile Leu Ile Phe
20 25

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 869 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Verticillium sp.
(B) STRAIN: CBS 830.95

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:43..738

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:133..738

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
CTCAATTCGT GAAAGTCTGA GATCAATTTT CAAGTTTGCA TT ATG AAG TTC CTT        54
                                                Met Lys Phe Leu
                                                -30

TAC ATT CTT CAG ACC CTA GCT ACC CTT GCG CTA GCC ACT CCC GTA CCT       102
Tyr Ile Leu Gln Thr Leu Ala Thr Leu Ala Leu Ala Thr Pro Val Pro
    -25                 -20                 -15

GAG ACG GTA CCA GAG AGT GAC CTG CAA AGT CGA GAA GAT TCT TTT GGT       150
Glu Thr Val Pro Glu Ser Asp Leu Gln Ser Arg Glu Asp Ser Phe Gly
-10                 -5                  1               5

ATA TCT TCT GTT CTC GTG CGT GAT GAA TTG ATC AAT GGT GGC GGC GCT       198
Ile Ser Ser Val Leu Val Arg Asp Glu Leu Ile Asn Gly Gly Gly Ala
            10                  15                  20

TGC CCC AAG GCT ATC CTC ATC TTT GCT CGA GGA ACG ATA GAA CTT GAT       246
Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg Gly Thr Ile Glu Leu Asp
        25                  30                  35

AAC ATG GGC TTA TTG GTT GGG CCA CCT CTT GCA GAC GGT CTA TCG GGT       294
Asn Met Gly Leu Leu Val Gly Pro Pro Leu Ala Asp Gly Leu Ser Gly
    40                  45                  50

ATC TTG GGT TCA AAA AAC CTC TGG GTC CAA GGC GTG GGT GGC CAA TAT       342
Ile Leu Gly Ser Lys Asn Leu Trp Val Gln Gly Val Gly Gly Gln Tyr
55                  60                  65                  70

GCT GCA AGC TTG GAG GGT AAT CTC TTT CCG GAT GGG ACC CCT CCT CAA       390
Ala Ala Ser Leu Glu Gly Asn Leu Phe Pro Asp Gly Thr Pro Pro Gln
                75                  80                  85

GCC ATC CAG GAG ATG ATT ACA TTG CTT CAA TTG GCG GAT ACT AAA TGT       438
Ala Ile Gln Glu Met Ile Thr Leu Leu Gln Leu Ala Asp Thr Lys Cys
            90                  95                  100

CCA AAC TCC AAG ATT GTC ACT GGG GGA TAT AGT CAA GGT GCT GCT CTC       486
Pro Asn Ser Lys Ile Val Thr Gly Gly Tyr Ser Gln Gly Ala Ala Leu
        105                 110                 115

GTG GCC GCA GCA ATT CGG GAT GTC AAG GCT TCG ATC CGA CAG AAG ATT       534
Val Ala Ala Ala Ile Arg Asp Val Lys Ala Ser Ile Arg Gln Lys Ile
    120                 125                 130

GTA GGA ACT GTA CTG TTC GGG TAC TCC AAA AAC AAA CAG AGG AAC GGT       582
Val Gly Thr Val Leu Phe Gly Tyr Ser Lys Asn Lys Gln Arg Asn Gly
135                 140                 145                 150

CAG GTA GAA AAC TAC TCT AAT GAC CGA CTC CGA GTT TAT TGC AAC CCT       630
Gln Val Glu Asn Tyr Ser Asn Asp Arg Leu Arg Val Tyr Cys Asn Pro
                155                 160                 165
```

```
GGG GAT TTA ATT TGC GAG GGG ACC TTG ATT GTT CTG CCA GTG CAC CTC.      678
Gly Asp Leu Ile Cys Glu Gly Thr Leu Ile Val Leu Pro Val His Leu
            170                 175                 180

CTT TAT GGA AAC CAA GCT TCT GGT CCT GCA GCA CAA TTC CTC GCT AGT      726
Leu Tyr Gly Asn Gln Ala Ser Gly Pro Ala Ala Gln Phe Leu Ala Ser
        185                 190                 195

AAG ATC AAT TCT TAGTTGAATT GTAGCCAACC GGATATGGCT GGGGATGGGC          778
Lys Ile Asn Ser
    200

CCAATCGTAA CCTATATAAT AGGCTTCATG CCATGTCTTA TTGCTAATAT ACGAAAAGAA    838

ATTCTGAATA CATAAAAAAA AAAAAAAAAA A                                   869
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Lys Phe Leu Tyr Ile Leu Gln Thr Leu Ala Thr Leu Ala Leu Ala
-30                 -25                 -20                 -15

Thr Pro Val Pro Glu Thr Val Pro Glu Ser Asp Leu Gln Ser Arg Glu
                -10                 -5                  1

Asp Ser Phe Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu Ile Asn
            5                   10                  15

Gly Gly Gly Ala Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg Gly Thr
        20                  25                  30

Ile Glu Leu Asp Asn Met Gly Leu Leu Val Gly Pro Pro Leu Ala Asp
    35                  40                  45                  50

Gly Leu Ser Gly Ile Leu Gly Ser Lys Asn Leu Trp Val Gln Gly Val
                55                  60                  65

Gly Gly Gln Tyr Ala Ala Ser Leu Glu Gly Asn Leu Phe Pro Asp Gly
            70                  75                  80

Thr Pro Pro Gln Ala Ile Gln Glu Met Ile Thr Leu Leu Gln Leu Ala
            85                  90                  95
```

```
Asp Thr Lys Cys Pro Asn Ser Lys Ile Val Thr Gly Gly Tyr Ser Gln
    100                 105                 110

Gly Ala Ala Leu Val Ala Ala Ala Ile Arg Asp Val Lys Ala Ser Ile
115                 120                 125                 130

Arg Gln Lys Ile Val Gly Thr Val Leu Phe Gly Tyr Ser Lys Asn Lys
                135                 140                 145

Gln Arg Asn Gly Gln Val Glu Asn Tyr Ser Asn Asp Arg Leu Arg Val
            150                 155                 160

Tyr Cys Asn Pro Gly Asp Leu Ile Cys Glu Gly Thr Leu Ile Val Leu
        165                 170                 175

Pro Val His Leu Leu Tyr Gly Asn Gln Ala Ser Gly Pro Ala Ala Gln
    180                 185                 190

Phe Leu Ala Ser Lys Ile Asn Ser
195                 200
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 999 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichophaea saccata
(B) STRAIN: CBS 804.70

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:68..763

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:161..763

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
CTTGGTACCG AGCTCGGATC CGAATTCGCA CAACGGGTTT CCGACTTTTG ATTTCCAACG      60

CTGCATC ATG AAG TTC CTC TAC GCC GTC CAG ACC TTA ATC GCC TTT GCA      109
        Met Lys Phe Leu Tyr Ala Val Gln Thr Leu Ile Ala Phe Ala
        -31 -30                 -25                 -20
```

```
CTG GCT ACG CCA GTG CCC GAG ACG GCT GTA GCA GTT GAT CTG CAG AAT      157
Leu Ala Thr Pro Val Pro Glu Thr Ala Val Ala Val Asp Leu Gln Asn
       -15                 -10                 -5

CGA GAA GAT TCT ATC GGC ATA TCC TCT GTC CTT GTG CGT GAT GAA CTG      205
Arg Glu Asp Ser Ile Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu
     1               5                  10                  15

CGC AAT GGC GGC GGC GCG TGT CCC AAG GCC ATT CTC ATC TTT GCT AGA      253
Arg Asn Gly Gly Gly Ala Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg
                20                  25                  30

GGT ACA ATG GAG CTG GAT AAC ATG GGC TTA TTA GTC GGG CCA GCT CTT      301
Gly Thr Met Glu Leu Asp Asn Met Gly Leu Leu Val Gly Pro Ala Leu
            35                  40                  45

GCA GGT GGC TTA GAG GCT ATG CTG GGT TCA AAT AAC CTC TGG GTC CAA      349
Ala Gly Gly Leu Glu Ala Met Leu Gly Ser Asn Asn Leu Trp Val Gln
        50                  55                  60

GGT GTA GGT GGC CAA TAT GCT GCC AAT CTC GAG GGC AAT CTA TTT CCA      397
Gly Val Gly Gly Gln Tyr Ala Ala Asn Leu Glu Gly Asn Leu Phe Pro
    65                  70                  75

GAT GGA ACA CCT CCC AAA GCC ATC CAG GAG ATG CTT AGT CTG CTC CAA      445
Asp Gly Thr Pro Pro Lys Ala Ile Gln Glu Met Leu Ser Leu Leu Gln
80                  85                  90                  95

TTA GCG GAC ACC AAG TGT CCA AAC TCT AAG ATT GTC ACA GGG GGG TAT      493
Leu Ala Asp Thr Lys Cys Pro Asn Ser Lys Ile Val Thr Gly Gly Tyr
                100                 105                 110

AGC CAA GGC GCT GCA CTC GTA GCC GCT GCT ATT CGC GAC GTC AAG GCT      541
Ser Gln Gly Ala Ala Leu Val Ala Ala Ala Ile Arg Asp Val Lys Ala
            115                 120                 125

TCC ATT CGA CAA AAG ATT GTA GGA ACT GTA CTC TTT GGG TAC ACC AAA      589
Ser Ile Arg Gln Lys Ile Val Gly Thr Val Leu Phe Gly Tyr Thr Lys
        130                 135                 140

AAC AAA CAG AAG AAC GGA CAG GTA GAA AAC TAC TCA ACT GAT CGA CTC      637
Asn Lys Gln Lys Asn Gly Gln Val Glu Asn Tyr Ser Thr Asp Arg Leu
    145                 150                 155

CGG GTT TAT TGC AAC GCC GGA GAC TTG ATT TGT CAA GGG ACC TTG ATT      685
Arg Val Tyr Cys Asn Ala Gly Asp Leu Ile Cys Gln Gly Thr Leu Ile
160                 165                 170                 175

GTT CTG CCA GCG CAT CTT CTT TAC GGA GAT CAG GCT GCT GGT CCA GCA      733
Val Leu Pro Ala His Leu Leu Tyr Gly Asp Gln Ala Ala Gly Pro Ala
                180                 185                 190
```

GCC CAG TTC CTT GCC AGC AAG ATC AGT TCA TAATTGCTTG ATCAACGCAT 783
Ala Gln Phe Leu Ala Ser Lys Ile Ser Ser
195 200

CACAGATTGC TGCCATGCAC CCATATATGG ATAGGAGAGA TCAAATATGG ACCTTACATA 843

GTCGCTCTAC CGCATCTGCT AAGAATATTT GATATTCCTT CGTTCCTTCT TAAGGCTAAT 903

GTATCCTCGA GATGGATGAT TAAGATCAGT ATAAAGAGAT GTAACAATTT ATACAGGCGA 963

TCTAGGTAGA TACTAAGACT ACATTTAAGT GTGAAA 999

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Lys Phe Leu Tyr Ala Val Gln Thr Leu Ile Ala Phe Ala Leu Ala
-31 -30 -25 -20

Thr Pro Val Pro Glu Thr Ala Val Ala Val Asp Leu Gln Asn Arg Glu
-15 -10 -5 1

Asp Ser Ile Gly Ile Ser Ser Val Leu Val Arg Asp Glu Leu Arg Asn
5 10 15

Gly Gly Gly Ala Cys Pro Lys Ala Ile Leu Ile Phe Ala Arg Gly Thr
20 25 30

Met Glu Leu Asp Asn Met Gly Leu Leu Val Gly Pro Ala Leu Ala Gly
35 40 45

Gly Leu Glu Ala Met Leu Gly Ser Asn Asn Leu Trp Val Gln Gly Val
50 55 60 65

Gly Gly Gln Tyr Ala Ala Asn Leu Glu Gly Asn Leu Phe Pro Asp Gly
70 75 80

Thr Pro Pro Lys Ala Ile Gln Glu Met Leu Ser Leu Leu Gln Leu Ala
85 90 95

Asp Thr Lys Cys Pro Asn Ser Lys Ile Val Thr Gly Gly Tyr Ser Gln
100 105 110

```
Gly Ala Ala Leu Val Ala Ala Ala Ile Arg Asp Val Lys Ala Ser Ile
    115                 120                 125

Arg Gln Lys Ile Val Gly Thr Val Leu Phe Gly Tyr Thr Lys Asn Lys
130                 135                 140                 145

Gln Lys Asn Gly Gln Val Glu Asn Tyr Ser Thr Asp Arg Leu Arg Val
                150                 155                 160

Tyr Cys Asn Ala Gly Asp Leu Ile Cys Gln Gly Thr Leu Ile Val Leu
            165                 170                 175

Pro Ala His Leu Leu Tyr Gly Asp Gln Ala Ala Gly Pro Ala Ala Gln
        180                 185                 190

Phe Leu Ala Ser Lys Ile Ser Ser
    195                 200
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 15-20 to page 11, lines 3-8.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
5 February, 1996	CBS 173.96

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Susan Kirkwood

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
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A. The indications made below relate to the microorganism referred to in the description on page 10, lines 15-20 to page 11, lines 3-8.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
22 December, 1995	CBS 830.95

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

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A. The indications made below relate to the microorganism referred to in the description on page 10, lines 15-20 to page 11, lines 3-8.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
January 22, 1996	CBS 126.96

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
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A. The indications made below relate to the microorganism referred to in the description on page 10, lines 15-20 to page 11, lines 3-8.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
January 22, 1996	CBS 127.96

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

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B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
15 March, 1996	DSM 10590

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, lines 15-22.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
15 March, 1996	DSM 10591

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, lines 15-22.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
27 November, 1996	DSM 11298

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Form PCT/RO/134 (July 1992)

CLAIMS

1. A lipolytic enzyme which is:

a) a polypeptide encoded by the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 10591, DSM 10590 or DSM 11298, or

b) a polypeptide produced by *Gliocladium sp.* CBS 173.96, *Verticillium sp.* CBS 830.95 or *Trichophaea* saccata CBS 804.70, or

c) a polypeptide having an amino acid sequence as shown in positions 1-200 of SEQ ID NO: 3, positions 1-202 of SEQ ID NO: 6, or positions 1-201 of SEQ ID NO: 8, or

d) an analogue of the polypeptide defined in (a), (b) or (c) which:

i) is at least 60% homologous with said polypeptide, or

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form.

2. The lipolytic enzyme of the preceding claim, which is obtainable from a microorganism, preferably a filamentous fungus, more preferably a strain of *Gliocladium*, *Verticillium* or *Trichophaea* (preferably *T. saccata*).

3. An alkaline lipolytic enzyme which is derivable from a strain of *Gliocladium* and has a lipolytic activity at pH 10 in the absence of Ca^{++} above 20% of the lipolytic activity at pH 10 in the presence of 50 mM Ca^{++}.

4. An alkaline lipolytic enzyme which is derivable from a strain of *Gliocladium* and gives a degree of hydrolysis above 15% on cotton/olive oil swatches in the Activity-in-Detergent (AiD) assay.

5. The lipolytic enzyme of any preceding claim, wherein the strain is *Gliocladium sp.* CBS 173.96 or belongs to *G. ammoniophilum*, *G. aureum*, *G. catenulatum*, *G. flavum*, *G. nigrovirens*, *G. roseum*, *G. sagariensis* or *G. solani*, preferably *Gliocladium sp.* CBS 173.96, *G. ammoniophilum* CBS 156.70, *G. aureum* IFO 9055, *G. catenulatum* NRRL 1091, *G. flavum* CBS 155.27, *G. nigrovirens* CBS 183.30, *G. roseum* CBS 126.96, *G. roseum* CBS 127.96, *G. sagariensis* IFO 9080 or *G. solani* CBS 707.86.

6. An alkaline lipolytic enzyme which is derivable from a strain of the genus *Verticillium* and retains more than 90% activity after 30 minutes incubation at pH 10.2, 40°C in a solution of 0.300 g/l C_{14}-C_{16} alkyl sulfate, 0.650 g/l alcohol ethoxylate (C_{12}-C_{14}, 6 EO), 1.750 zeolite P, 0.145 g/l Na_2CO_3, 0.020 g/l acrylate/maleate copolymer and 0.050 g/l carboxymethyl cellulose.

7. The lipolytic enzyme of the preceding claim wherein the strain is *Verticillium sp.* CBS 830.95.

8. The lipolytic enzyme of any preceding claim which has lipase and/or cutinase activity.

9. The lipolytic enzyme of any preceding claim in the form of an enzymatic detergent additive which is a non-dusting granulate, a stabilized liquid, a slurry, or a protected enzyme.

10. An enzymatic detergent composition comprising a surfactant and the lipolytic enzyme of any preceding claim.

11. A method of producing an alkaline lipolytic enzyme, comprising cultivation of a lipolytic enzyme-producing strain of *Gliocladium*, *Verticillium* or *Trichophaea* in a suitable nutrient medium, followed by recovery of the alkaline lipolytic enzyme.

12. The method of the preceding claim, wherein the strain is *Gliocladium sp.* CBS 173.96 or belongs to *G. ammoniophilum*, *G. aureum*, *G. catenulatum*, *G. flavum*, *G. nigrovirens*, *G. roseum*, *G. sagariensis* or *G. solani*, preferably *Gliocladium sp.* CBS 173.96, *G. ammoniophilum* CBS 156.70, *G. aureum* IFO 9055, *G. catenulatum* NRRL 1091, *G. flavum* CBS 155.27, *G. nigrovirens* CBS 183.30, *G. roseum* CBS 126.96, *G. roseum* CBS 127.96, *G. sagariensis* IFO 9080 or *G. solani* CBS 707.86.

13. The method of claim 13, wherein the strain is a strain of *Verticillium*, and is preferably *Verticillium sp.* CBS 830.95.

14. The method of claim 13, wherein the strain is a strain of *Trichophaea*, preferably *T. saccata*, most preferably *T. saccata* CBS 804.70.

15. A method for producing an alkaline lipolytic enzyme, comprising:

a) isolating a DNA sequence encoding the lipolytic enzyme from a lipolytic enzyme-producing strain of *Gliocladium*, *Verticillium* or *Trichophaea*,

b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,

c) transforming a suitable heterologous host organism with the vector,

d) cultivating the transformed host organism under conditions leading to expression of the lipolytic enzyme, and

e) recovering the lipolytic enzyme from the culture medium.

16. The method of the preceding claim, wherein the host organism is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of *Aspergillus*, more preferably *A. oryzae*.

17. The method of any of claims 13-16, wherein the DNA sequence is isolated by a method comprising:

a) cloning, in suitable vectors, a cDNA library from the lipolytic enzyme-producing strain of *Gliocladium*, *Verticillium* or *Trichophaea*,

b) transforming suitable yeast host cells with said vectors,

c) cultivating the transformed yeast host cells under suitable conditions to express the alkaline lipolytic enzyme,

d) screening for positive clones by determining the lipolytic enzyme activity expressed in step (c).

18. The method of any of claims 13-17, wherein the the lipolytic enzyme producing strain is *Gliocladium sp.* CBS 173.96 or belongs to *G. ammoniophilum*, *G. aureum*, *G. catenulatum*, *G. flavum*, *G. nigrovirens*, *G. roseum*, *G. sagariensis* or *G. solani*, preferably *Gliocladium sp.* CBS 173.96, *G. ammoniophilum* CBS 156.70, *G. aureum* IFO 9055, *G. catenulatum* NRRL 1091, *G. flavum* CBS 155.27, *G. nigrovirens* CBS 183.30, *G. roseum* CBS 126.96, *G. roseum* CBS 127.96, *G. sagariensis* IFO 9080 or *G. solani* CBS 707.86.

19. The method of any of claims 11-15, wherein the lipolytic enzyme producing strain belongs to*Verticillium* and is preferably *Verticillium sp.* CBS 830.95.

20. The method of any of claims 11-15 wherein the lipolytic enzyme producing strain belongs to *Trichophaea*, preferably *T. saccata*, most preferably CBS 804.70.

21. An isolated DNA sequence which encodes the lipolytic enzyme of any of claims 1-7.

22. An isolated, lipolytic enzyme encoding DNA sequence which comprises:

a) the lipolytic enzyme encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* DSM 10591, DSM 10590 or DSM 11298, or

b) the DNA sequence shown in positions 114-713 of SEQ ID NO: 2, positions 133-738 of SEQ ID NO: 5 or positions 161-763 of SEQ ID NO: 7, or

c) an analogue of the DNA sequence defined in a) or b) which

i) is at least 60% homologous with said DNA sequence, or

ii) hybridizes with said DNA sequence at 55°C.

23. The DNA sequence of claim 19 or 20, wherein the lipolytic enzyme-encoding sequence is obtainable from a microorganism, preferably a filamentous fungus.

24. The DNA sequence of the preceding claim, wherein the filamentous fungus is a strain of *Gliocladium*, *Verticillium* or *Trichophaea*, preferably the strain *Gliocladium sp.* CBS 173.96, *Verticillium sp.* CBS 173.96 or *T. saccata* CBS 804.70.

25. A recombinant expression vector comprising the DNA sequence of any of claims 19-24.

26. A cell comprising the DNA sequence of any of claims 19-24 or the recombinant expression vector of claim 25.

27. The cell of the preceding claim, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of *Aspergillus*, preferably *A. oryzae*.

28. A method of producing a lipolytic enzyme, comprising culturing the cell of any of claims 26-29 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

29. A biologically pure culture of a microbial strain which belongs to the genus *Gliocladium* or *Verticillium* and is capable of producing an alkaline lipolytic enzyme.

30. The culture of the preceding claim wherein the strain is *Gliocladium sp.* CBS 173.96, *Gliocladium roseum* CBS 126.96 or 127.96 or *Verticillium sp.* CBS 830.95.

31. *Escherichia coli* strain DSM 10591, DSM 10590 or DSM 11298 or a mutant thereof having lipolytic enzyme encoding capability.

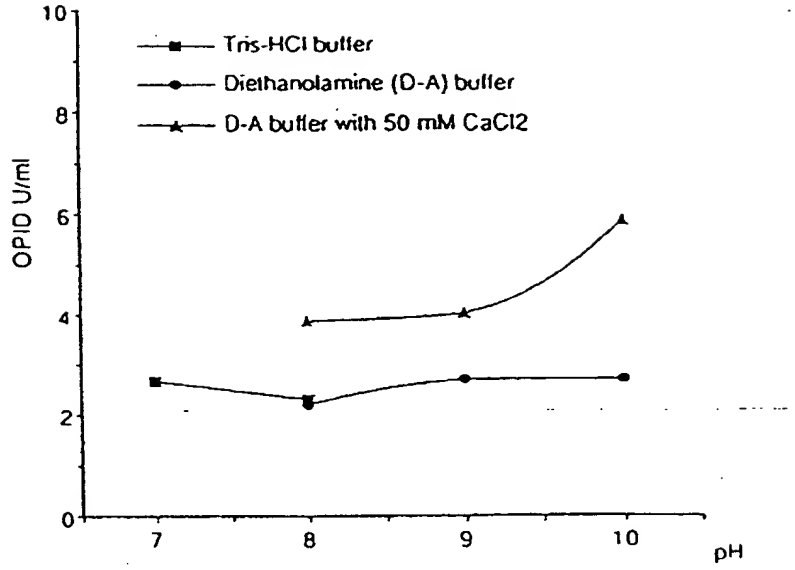

FIG. 1: Gliocladium sp. NN140631

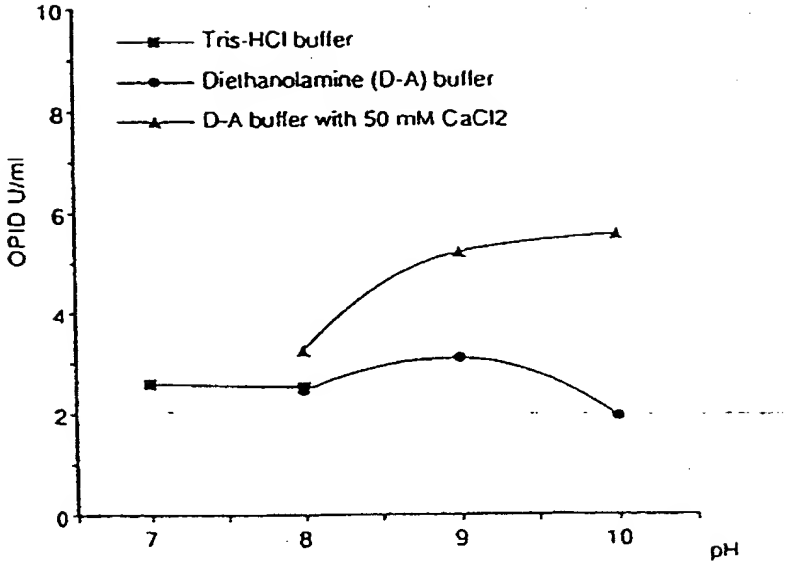

FIG. 2: G. solani NN102998

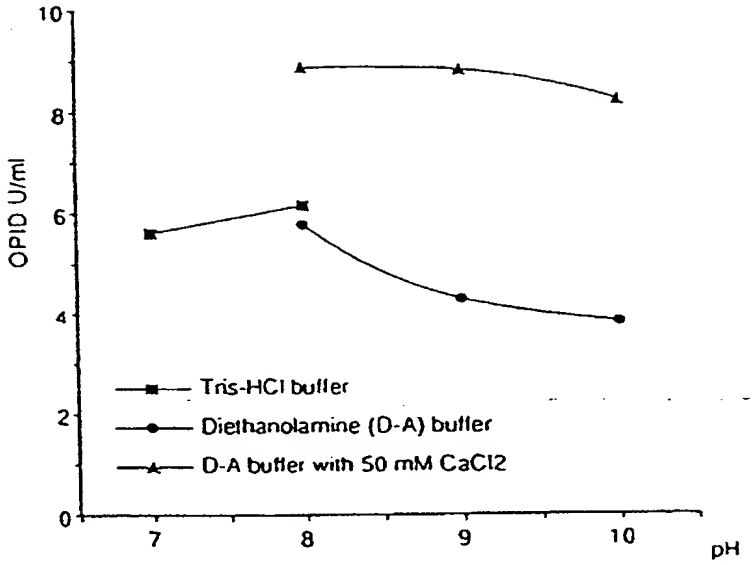

FIG. 3: G. roseum NN141784

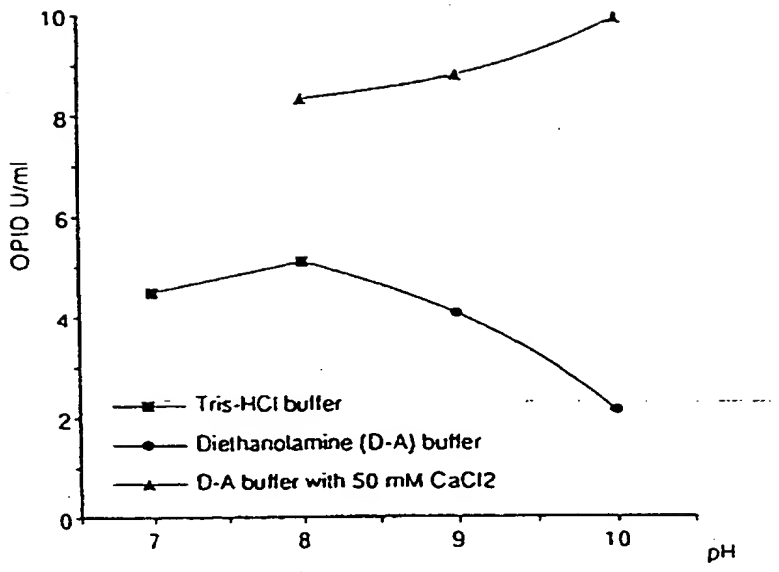

FIG. 4: G. aureum NN102987

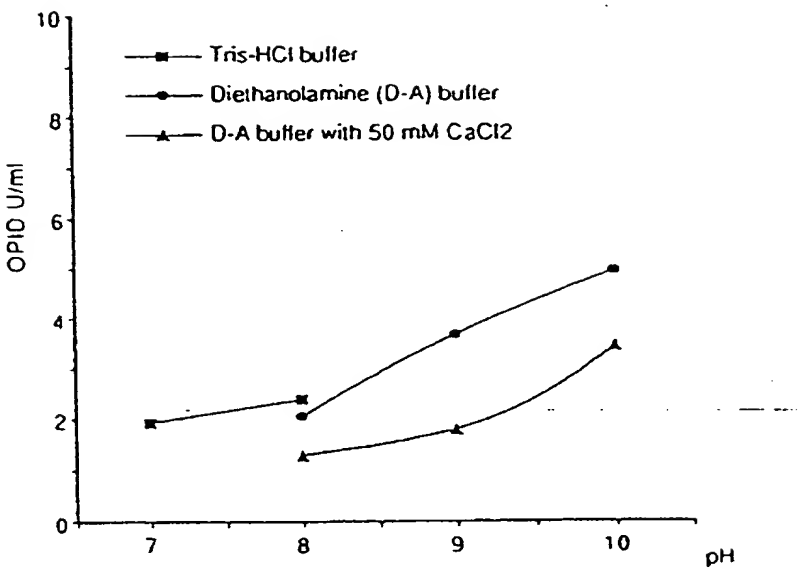

FIG. 5: G. roseum NN141961

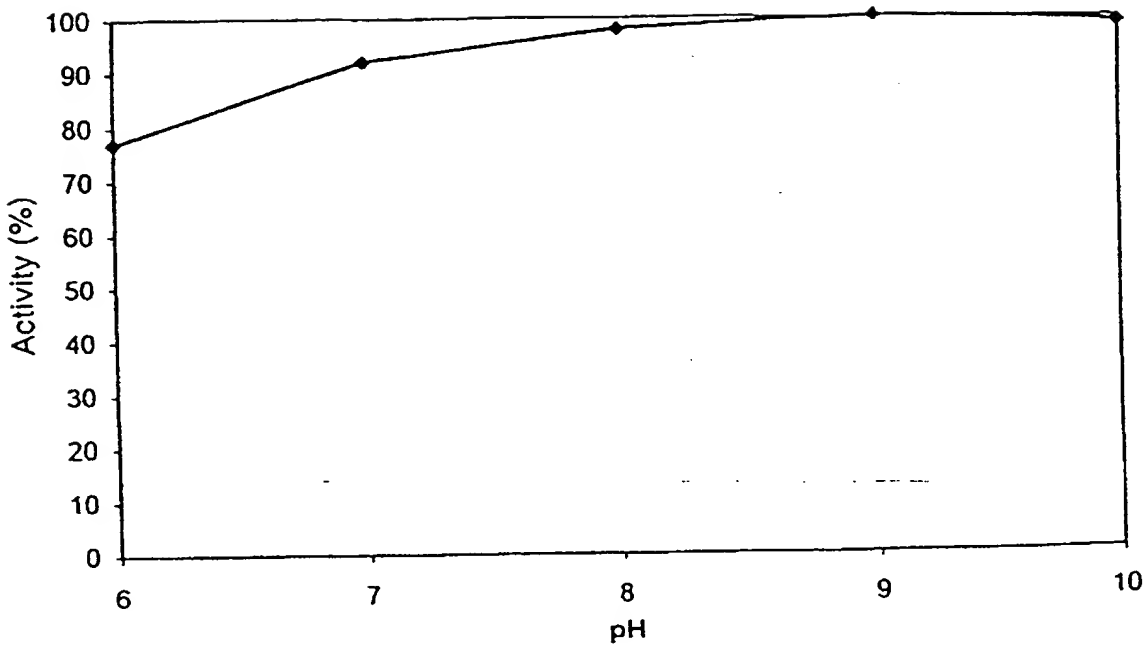

FIG. 6: Verticillium sp. CBS 830.95

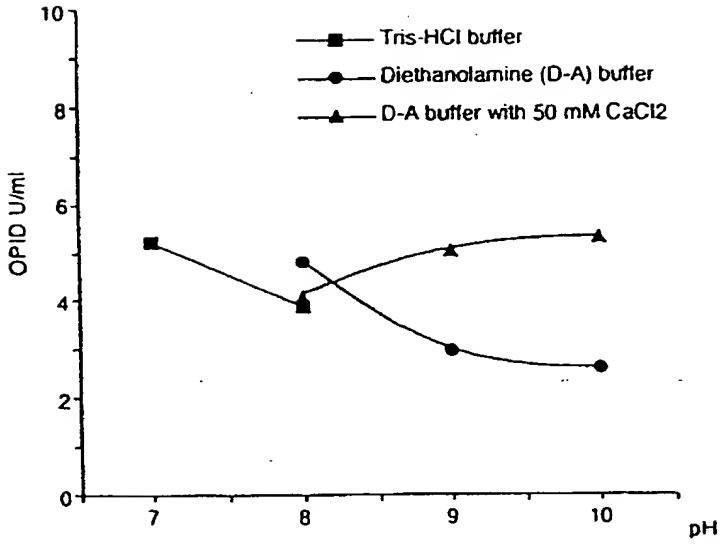

FIG. 7: T. saccata CBS 804.70

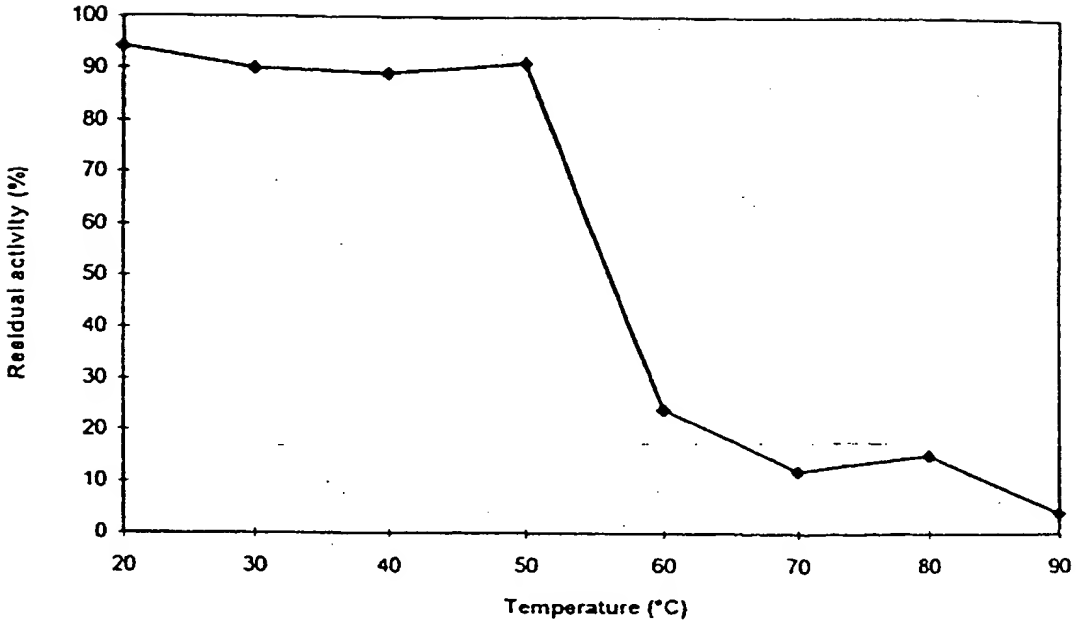

FIG. 8: Verticillium sp. CBS 830.95

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00179

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/20, C12N 1/21 // C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, DBA, CA, MEDLINE, EMBL/GENBANK/SWISSPROT/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9414964 A1 (UNILEVER PLC), 7 July 1994 (07.07.94), fig 1D, claim 10 --	1-31
X	WO 9414940 A1 (NOVO NORDISK A/S), 7 July 1994 (07.07.94), claims and the whole document --	1-31
X	EP 0218272 A1 (GIST-BROCADES N.V.), 15 April 1987 (15.04.87), column 15, line 39-41, claims --	1-31
P,X	WO 9613578 A1 (NOVO NORDISK A/S), 9 May 1996 (09.05.96), page 1, line 19-22, claim and the whole document --	1-31

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 1997	04 -08- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00179

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	Dialog Information Service, File 55, BIOSIS PREVIEWS, Dialog accession no. 10078966, BIOSIS no. 95078966, Van Tilburg A-U B et al: "Production of extracellular proteins by the biocontrol fungus gliocladium-virens"; & Appl Environ Microbiol 59 (1). 1993. 236-242 --	1-31

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Information on patent family members

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